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Tumour Review

Testicular cancer: Determinants of cisplatin sensitivity and novel therapeutic opportunities



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ABSTRACT

Testicular cancer (TC) is the most common solid tumor among men aged between 15 and 40 years. TCs are highly aneuploid and the 12p isochromosome is the most frequent chromosomal abnormality. The mutation rate of TC is low, with recurrent mutations in *KIT* and *KRAS* observed only at low frequency in seminomas. Overall cure rates are high, even in a metastatic setting, resulting from excellent cisplatin sensitivity of TCs. Factors contributing to the observed cisplatin sensitivity include defective DNA damage repair and a hypersensitive apoptotic response to DNA damage. Nonetheless, around 10–20% of TC patients with metastatic disease cannot be cured by cisplatin-based chemotherapy. Resistance mechanisms include downregulation of OCT4 and failure to induce PUMA and NOXA, elevated levels of MDM2, and hyperactivity of the PI3K/AKT/mTOR pathway. Several pre-clinical approaches have proven successful in overcoming cisplatin resistance, including specific targeting of PARP, MDM2 or AKT/mTOR combined with cisplatin. Finally, patient-derived xenograft models hold potential for mechanistic studies and pre-clinical validation of novel therapeutic strategies in TC. While clinical trials investigating targeted drugs have been disappointing, pre-clinical successes with chemotherapy and targeted drug combinations fuel the need for further investigation in clinical setting.

Clinical and pathological characteristics of TC

Testicular cancer (TC) accounts for approximately 1% of all cancers in men worldwide, while it is the most common solid tumor among young men (15–40 years) [1]. Incidence of TC varies widely across different regions, with highest incidence in Northern Europe and very low incidence in Central Africa [1]. Incidence rates of TC have increased since the 1960s in Northern European countries, affecting age groups older than 15 years with no proven causative mechanisms identified so far [2].

Germ cell tumor (GCT) is the predominant histology of TC patients (95%), divided into seminomas and non-seminomas, of which seminomas are slightly more common than non-seminomas [3]. GCTs arise from gonocytes that fail to differentiate into spermatogonias, and the pluripotency of these cells allows them to develop into highly diverse histological tumors. Seminomas are blocked in the earliest differentiation state whereas non-seminomas consist of diverse histological subtypes with a varying degree of differentiation: embryonal carcinoma (EC) (undifferentiated), choriocarcinoma (CC), yolk sac carcinoma

(YSC) or teratoma (mature and immature) [4]. Mixed GCTs are common and may appear in any form.

Diagnosis of TC is performed by clinical evaluation, ultrasound examination of the testes, orchiectomy of the involved testicle and determination of serum tumor markers. These tumor biomarkers include LDH (lactate dehydrogenase), AFP (alpha-fetoprotein) and β -HCG (β -human chorionic gonadotropin), and assist in the diagnosis [seminoma vs non-seminoma], staging, risk stratification and follow-up after treatment of TC patients [5]. The international clinical staging system recommended for TC is the Tumor, Node, Metastasis (TNM) classification of the International Union Against Cancer (UICC) [6]. Current risk stratification was established in 1997 by the International Germ Cell Consensus Classification (IGCCC) and constitutes a prognostic staging system for patients with disseminated disease [7].

Overall cure rate of TC is very good, also in a metastatic setting, which is mostly resulting from the high sensitivity of TCs to the chemotherapeutic drug cisplatin. The 5-year relative survival rates for seminoma and non-seminoma patients are above 90% [2]. For patients with stage I disease the survival rate is even somewhat higher exceeding

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Table 1
Risk stratification for metastatic patients [7].

Risk group	Non-seminoma	Survival	Seminoma	
	Spread and tumor markers		Spread and tumor markers	Survival
Good prognosis	<ul style="list-style-type: none"> • Testis or retroperitoneal primary • No metastasis to organs other than the lungs and/or lymph nodes • Tumor markers level normal or at least 1 tumor marker above normal: <ul style="list-style-type: none"> • LDH < 1.5x ULN • beta-hCG < 5000 mIU/mL • AFP < 1000 ng/mL 	56% of patients 5-year PFS: 89% 5-year OS: 92%	<ul style="list-style-type: none"> • Any primary site • No metastasis to organs other than the lungs and/or lymph nodes • Normal AFP • Any beta-hCG and LDH 	90% of patients 5-year PFS: 82% 5-year OS: 86%
Intermediate prognosis	<ul style="list-style-type: none"> • Testis or retroperitoneal primary • No metastasis to organs other than the lungs and/or lymph nodes • At least 1 tumor marker substantially above normal: <ul style="list-style-type: none"> • LDH 1.5 – 10x ULN • beta-hCG ≥ 5000 ≤ 50000 mIU/mL • AFP ≥ 1000 ≤ 10000 ng/mL 	28% of patients 5-year PFS: 75% 5-year OS: 80%	<ul style="list-style-type: none"> • Any primary site • Metastasis to organs other than the lungs and/or lymph nodes • Normal AFP • Any beta-hCG and LDH 	10% of patients 5-year PFS: 67% 5-year OS: 72%
Poor prognosis	<ul style="list-style-type: none"> • Mediastinal primary with or without metastases • Metastasis to organs other than the lungs and/or lymph nodes • At least 1 or more tumor marker levels highly elevated: <ul style="list-style-type: none"> • LDH > 10x ULN • beta-hCG > 50000 mIU/mL • AFP > 10000 ng/mL 	16% of patients 5-year PFS: 41% 5-year OS: 48%	No poor prognosis patients	
			No poor prognosis patients	

LDH: lactate dehydrogenase, hCG: human chorionic gonadotropin, AFP: alpha-fetoprotein, ULN: upper limit of normal, PFS: progression free survival, OS: overall survival.

95% regardless of tumor type. However, patients with metastatic disease have poorer survival outcomes depending on the tumor type and extent of disease and prognosis group. Seminoma and non-seminoma tumors have different treatment sensitivity and invasive capabilities. At moment of diagnosis 25% of seminoma patients have metastatic disease, while 60% of non-seminoma patients have metastatic lesions [8]. The IGCCC classification stratifies patients into good, intermediate and poor prognosis groups which are determined based on clinical features, including the location of the metastases and the levels of different tumor biomarkers [7]. According to the IGCCC there are no advanced seminoma patients within the poor prognosis group, related to their better overall response to chemotherapy. Staging, risk stratification and 5-year survival rates for both seminoma and non-seminoma patients are summarized in Table 1.

Late relapses (LR) occur in 1% to 6% of TC patients and are considered to be more difficult to treat than early relapses [9]. A pooled analysis of several studies has shown that late relapses occur more often in non-seminomas (3.2%) than in seminomas (1.4%), with teratoma as the most frequent histological component (60%), followed by YSC (47%) [9,10]. Because of the high rate of teratoma and teratoma with malignant transformation to non-germ cell malignancies (20%), LR are less likely to respond to chemotherapy [9]. Additionally, YSC has been associated with poor prognosis, this could partly explain the worse clinical outcome associated with late relapse of this histological subtype [11].

Standard treatment of TC

Treatment of TC depends on stage and tumor type, i.e. seminoma or non-seminoma. Stage I or localized disease, either seminoma or non-seminoma, is treated with orchiectomy followed by active surveillance or with adjuvant treatment based on risk factors and patient preference. Patients with stage IIA/B seminoma are treated with orchiectomy followed by either radiotherapy or chemotherapy, and bulky stage IIC and III seminoma patients receive chemotherapy after orchiectomy. Patients with disseminated non-seminoma (intermediate or poor risk IGCCC) are treated with four courses of the BEP (bleomycin, etoposide and

cisplatin) or VIP (etoposide, ifosfamide and cisplatin) regimen, after surgical removal of the affected testicle. In case of residual disease after completion of chemotherapy patients undergo a surgical removal of affected lymph nodes and/or metastases that had not completely disappeared after chemotherapy. Approximately 10–15% of patients with disseminated disease will need second-line treatment as a consequence of relapse or refractory disease [12]. Various effective salvage strategies are currently available, including standard-dose and high-dose chemotherapy regimens. The choice of standard-dose salvage treatment depends on which drugs were initially used in combination with cisplatin. Some common and effective standard-dose salvage treatments have been reported with long-term remissions ranging from 23 to 54% using VIP (cisplatin, ifosfamide and etoposide) [13,14], 63% using TIP (paclitaxel, ifosfamide and cisplatin) [15], 24% using VeIP (vinblastine, ifosfamide and cisplatin) [16] and 51% using GIP (gemcitabine, ifosfamide and cisplatin) [17]. High-dose chemotherapy with carboplatin and etoposide with autologous bone marrow transplantation was initially studied in patients with a predicted poor outcome to standard-dose salvage treatment or patients who had already failed a prior standard-dose salvage treatment. Initial results were exceptional with a complete response rate of 26% [18]. More recently, high-dose chemotherapy with autologous peripheral stem cell transplantation has shown long-term remissions in 63% of patients, including patients who received this as third-line treatment [19]. As demonstrated by the data presented above, standard-dose and high-dose salvage chemotherapy are both able to overcome cisplatin resistance. A large retrospective study suggested that high-dose chemotherapy is superior to standard-dose chemotherapy as first salvage treatment [20]. To date, it remains unclear whether standard-dose or high-dose salvage chemotherapy provides better outcomes when used as initial salvage treatment. To address this question, a prospective global trial (the TIGER trial) is currently performed in TC patients with relapsed or refractory disease. In this trial, patients are being randomized between 4 courses of TIP vs. TI-CE [with 3 courses of high dose carboplatin-etoposide with an autologous peripheral stem-cell transplantation] (NCT02375204).

Genetic alterations in TC patients

TCs are highly aneuploid and frequently show large scale copy number gains and losses [21–23]. The most common anomaly is the presence of a 12p isochromosome (i(12p)) affecting more than 80% of TCs [24]. This region of the small arm of chromosome 12 contains the *KRAS* proto-oncogene, as well as some stem cell-related genes including *NANOG* and *STELLAR*. Of note, pre-malignant lesions do not contain i(12p) [25], suggesting that this genetic event is not an early event in the development of TC. *KIT* gene amplifications at 4q12 were identified in 21% of seminomas and 9% of non-seminomas [26]. Copy number gains of the *KIT* gene were associated with an increased expression of the *KIT* protein, although other mechanisms besides copy number gain can also result in increased *KIT* expression [26].

Despite the high levels of aneuploidy of TCs, the somatic mutation rate is low. Whole exome sequencing revealed that, on average, TC tumors have a mutation rate of 0.51 somatic mutations per Mb, while lung cancers carry 8.0 mutations/Mb, and melanomas display 11.0 mutations/Mb [27]. The most frequently mutated gene in TC is *KIT*, and has been observed predominantly in seminomas [27,28]. The proportion of *KIT* hotspot mutations in seminomas ranged from 18% to 31% [27,29]. Besides *KIT*, mutations in *KRAS* and *NRAS* have been reported, albeit with a lower frequency [27,30]. Other mutations that were recently described include: *CDC27*, a potential tumor suppressor gene [27,31]; *RPL5*, a ribosomal protein [30]; and *RAC1*, a member of the Rho family of GTPases [32].

Studies looking specifically at mutations in cisplatin-resistant patients have also been performed. Results showed a higher incidence of activating mutations in *RAS*, *PIK3CA* and *AKT1* in chemoresistant TCs [33]. Mutations in *XRCC2* have been reported in 5 cases of refractory TCs [27,34]. *XRCC2* belongs to the *RAD51* protein family and participates in DNA repair through homologous recombination. *XRCC2* interacts with other *RAD51* protein family members and facilitates the assembly of *RAD51* onto DNA double-stranded breaks. Interestingly, *XRCC2* variants were shown to confer resistance to cisplatin-induced DNA damage and have been associated with breast cancer risk and survival [35,36].

One of the characteristics of TC is the presence of wild type (WT) *TP53* in the majority of patients, although mutations in *TP53* have been observed in a few cases [37]. Activity of p53 is regulated by the E3 ubiquitin ligase *MDM2*, which prevents transcription of target genes by p53 through direct binding to the transactivation domain of p53 and through targeting p53 for proteasomal degradation by ubiquitination [38]. *MDM2* itself is a transcriptional target of p53, which provides an autoregulatory negative feedback to maintain p53 levels low under physiological conditions. Bagrodia *et al.* showed that the incidence of p53 pathway mutations was higher in chemoresistant TC compared to chemosensitive TC, with *TP53* mutations mainly occurring in non-seminoma [32]. *MDM2* and *MYCN* amplifications, both affecting p53 signaling, have also been described in chemoresistant and refractory patients [32,34]. As *TP53* mutations are rare, *MDM2* amplification is likely a selection mechanism to prevent cell cycle arrest and DNA repair during the progression of disease.

Cisplatin sensitivity

With the introduction of cisplatin-based chemotherapy in the 1970s, cure rates of metastatic TC have improved drastically [39]. Even though cisplatin has been used in the clinic for a long time, the mechanisms behind the excellent sensitivity of these tumor types remain elusive. Two features of TC tumors that have been related to cisplatin sensitivity of TC are: insufficient DNA repair of cisplatin-induced DNA damage, and a hypersensitive apoptotic response, both discussed below and summarized in Figs. 1 and 2.

Cisplatin interacts with DNA bases generating different forms of DNA adducts that are predominantly intrastrand cross-links (> 90%)

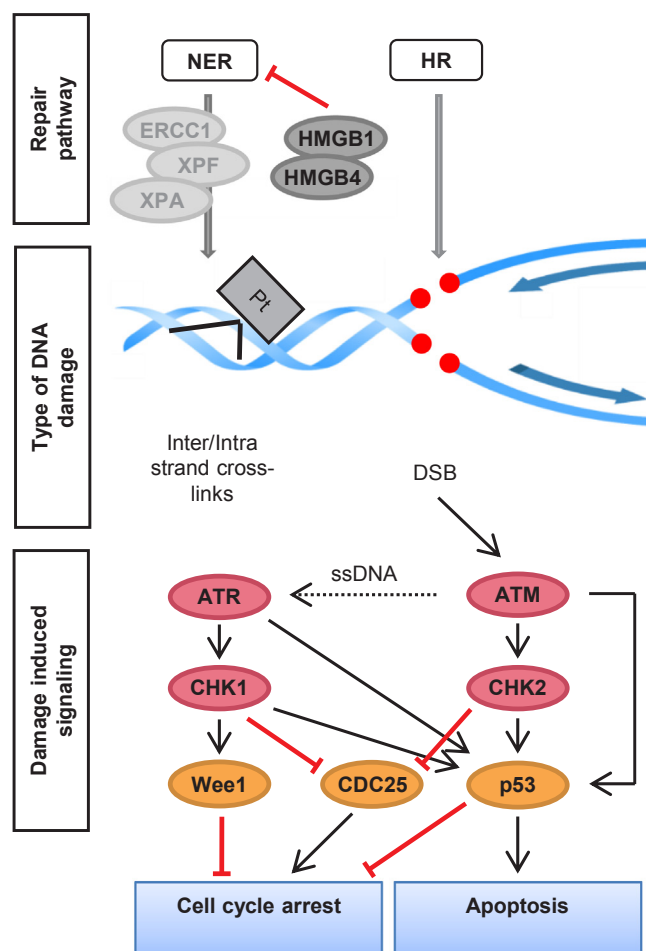


Fig. 1. Overview of cisplatin induced DNA damage and repair. Cisplatin treatment mainly induces intra- and interstrand DNA cross-links. These lesions are repaired by NER. Low expression of NER related proteins (e.g. ERCC1, XPF and XPA) are a rate limiting factor for cisplatin-DNA cross-link repair. High expression of HMGB1/4 can inhibit NER. Due to less functional NER, unrepaired lesions may further develop into DSB which require HR for repair. ATM is recruited to sites of DSBs and leads to a DNA damage induced signaling cascade that activates a p53-dependent transcriptional program leading to cell cycle arrest or apoptosis. HR requires DNA-end resection that produces tracts of single-stranded DNA (ssDNA). ATR is recruited to tracts of ssDNA, where it phosphorylates and activates CHK1 leading to activation of Wee1 and proteasomal degradation of CDC25, thereby regulating cell cycle arrest.

but also interstrand cross-links, protein-DNA cross-links and DNA mono-adducts [40,41]. Cisplatin-induced DNA cross-links disrupt the structure of the DNA which can be recognized by DNA repair proteins. Cisplatin-induced intrastrand crosslinks are mostly repaired by the nucleotide excision repair (NER) pathway [42]. However, it has been shown that various high mobility group (HMG) proteins bind to DNA adducts inhibiting NER [43,44]. In addition, some essential proteins involved in NER including ERCC1, XPF and XPA were shown to have low expression levels in TC [45,46]. Low levels of ERCC1 and XPF, while still sufficient to perform NER, are rate-limiting for the repair of cisplatin-DNA cross-links in TC cells, contributing to the excellent sensitivity to cisplatin treatment [47]. Furthermore, cytotoxicity of cisplatin can be partially explained by defective double strand break (DSB) repair by homologous recombination. If DNA crosslinks are not repaired they can lead to collapsed replication forks, which results in the formation of DSBs. Interestingly, in a panel of TC cell lines, all models were highly sensitive to the combination of cisplatin with PARP inhibitors, providing a potential therapeutic approach for resistant TC

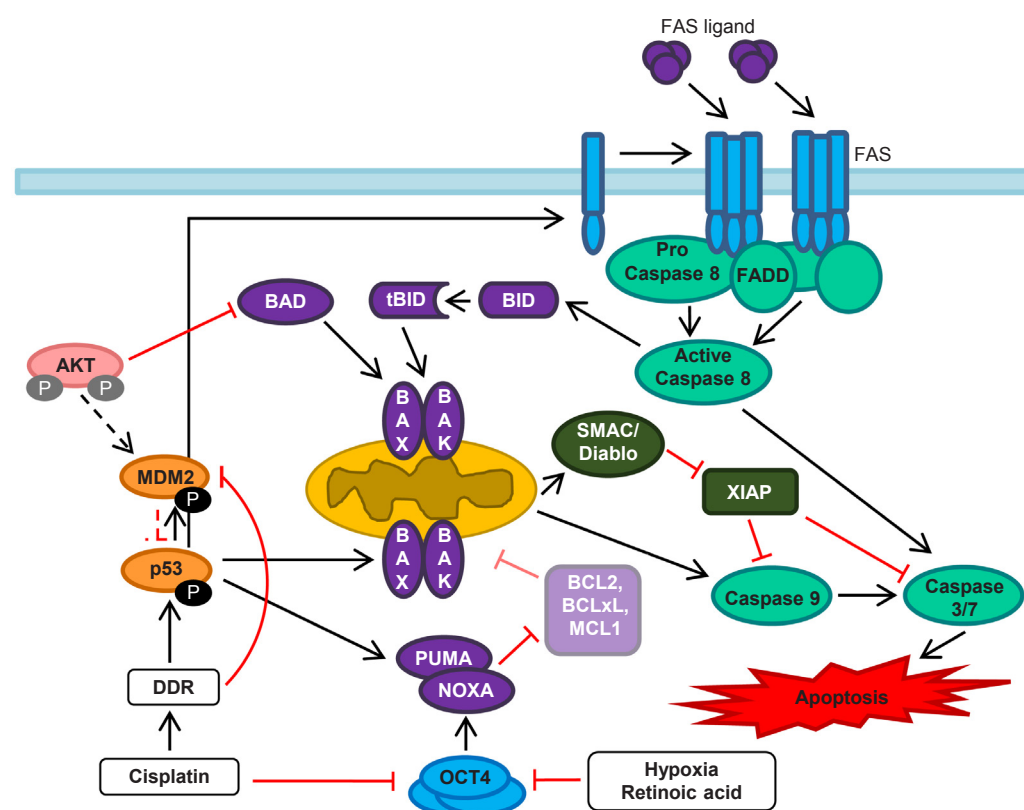


Fig. 2. Apoptotic signaling in cisplatin sensitive TC. Apoptosis is regulated at several levels with a key role for p53. Cisplatin treatment activates the DDR leading to p53 activation. P53 transcriptionally regulates proteins involved in both the extrinsic and intrinsic apoptosis pathway, including FAS death receptor, BAX, PUMA and NOXA. Activated p53 enhances expression of the FAS death receptor. Binding of FAS ligand to FAS death receptor leads to formation of the death-inducing signaling complex (DISC) including FADD and procaspase-8, cleavage of procaspase-8 and subsequently activation of a caspase cascade leading to apoptosis. The intrinsic apoptosis pathway is regulated by pro- and anti-apoptotic proteins. Pro-apoptotic proteins either directly facilitate BAX and BAK oligomerization in the mitochondrial outer membrane, or indirectly by inhibiting anti-apoptotic proteins. Mitochondrial outer membrane permeabilization leads to release of SMAC/Diablo and activation of a caspase cascade. SMAC/Diablo represses the caspase inhibitor XIAP. Crosstalk between the extrinsic and intrinsic apoptosis pathways exists in form of caspase-8 mediated cleavage of BID. High levels of OCT are correlated with high NOXA protein expression.

tumors [48]. The DNA damage response (DDR) machinery is activated in response to DSBs and involves recruitment of ATM to DSBs and activation of ATM signaling. ATM activation leads to phosphorylation of ~700 substrates, including MDM2 and p53 [49]. As a consequence, p53 is no longer ubiquitinated by MDM2, leading to its stabilization and full activation [50] (Fig. 1). Transactivation of the p53 target gene *CDKN1A*, encoding the CDK inhibitor p21, is crucial for the induction of cell cycle arrest in G1 phase. Apoptosis on the other hand, can be triggered by the p53-mediated transcription of pro-apoptotic BCL-2 family members including *BBC3* (PUMA) and *PMAIP1* (NOXA) [51].

Activation of the DDR by cisplatin leads to a rapid induction of apoptosis with a major role for WT p53 in TC. Mutations in *TP53* are rarely observed in TC. Nevertheless, *TP53* alterations have been detected in a small subset (~15%) of cisplatin-resistant or relapsed TC patients [32,37]. Of note, no correlation has been found between p53 protein expression levels and chemosensitivity [52]. In fact, chemoresistant tumors exhibited a trend towards higher positivity for both p53 and MDM2 compared to cisplatin-sensitive tumors, suggesting increased importance of MDM2-mediated inhibition of p53 activity. This hypothesis is supported by functional studies using cisplatin-sensitive and -resistant TC cell lines indicating that the interaction between p53 and MDM2 required higher doses of cisplatin to be disrupted in resistant cell lines [53].

Cisplatin treatment results in enhanced expression of the FAS death receptor, a transcriptional target of p53, and subsequent activation of the extrinsic apoptosis pathway via the interaction between FAS and FAS ligand [53,54]. Other studies showed activation of the intrinsic apoptosis pathway, where cisplatin led to increased expression of pro-apoptotic proteins PUMA and NOXA which are involved in the regulation of the intrinsic apoptosis pathway [55–57]. In addition, high protein levels of the pluripotency factor OCT4, typical for the EC subtype, strongly correlated with a high expression of NOXA, while OCT4 knockdown resulted in a strong reduction of NOXA [57]. Interestingly,

high NOXA expression has been correlated with good prognosis in TC tumors with EC histology [58]. Crosstalk between the extrinsic and intrinsic apoptosis pathways, where FAS receptor activation results in caspase-8-mediated cleavage of BID, may further strengthen the apoptotic response (Fig. 2). A recent study proposed that TC tumors with WT *TP53* have an intrinsically heightened apoptotic potential caused by their increased mitochondrial priming [30].

The difference in cisplatin-sensitivity between seminoma and non-seminoma tumors could be explained by a lower level of DNA methylation in seminomas [29,59,60]. An interesting hypothesis postulated in the study by Shen *et al.*, is that activating *KIT* mutations, predominantly observed in seminomas, induce a primordial quiescent state in seminoma cells that suppresses the expression of genes involved in methylation, including *UHRF1* and *DNMT1* [29]. Consistently, methylation levels have been linked to the differentiation status of germ cell tumors as well as to cisplatin-sensitivity in vitro [61].

Cisplatin resistance in TC and novel pre-clinical targets

While TC is a highly curable disease, partly caused by mechanisms outlined above, approximately 10–15% of patients develop a tumor relapse after initial treatment or have refractory disease [12]. Several mechanisms underlying cisplatin resistance have been identified, suggesting no uniform cause of resistance. Some of the most prominent resistance mechanisms that potentially have therapeutic value, will be discussed here (Fig. 3).

As described above, the excellent sensitivity of TC tumors to cisplatin treatment can be attributed to diminished capacity to repair cisplatin-induced DNA cross-links. Consequently, cisplatin resistance may arise from an enhanced ability to resolve DNA damage, or by increased coping mechanisms towards unrepaired DNA lesions. Increased expression of ERCC1 in ovarian cancer cell lines has been associated with cisplatin resistance [62]. However, there are currently no data that

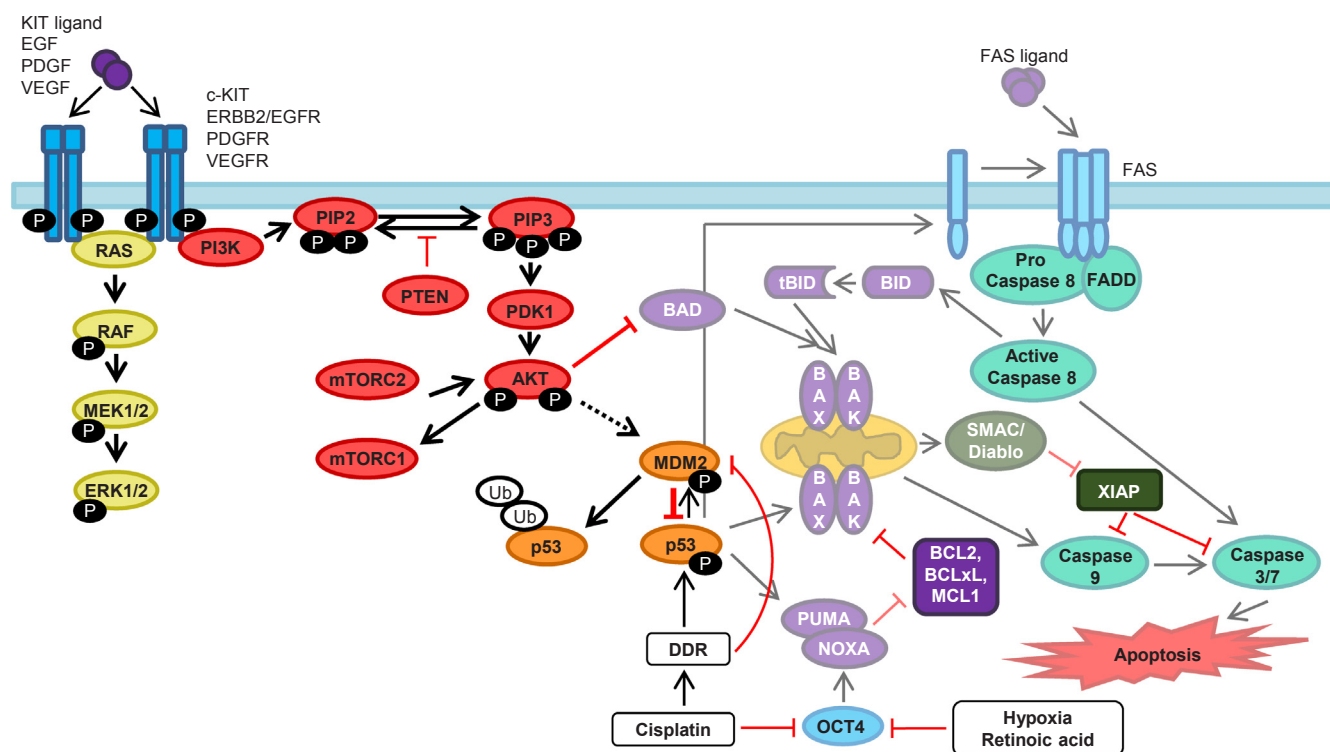


Fig. 3. Activation of cell survival signaling and lower apoptosis induction factors of cisplatin resistance in TC. Growth factor binding to different receptor tyrosine kinases (RTK) leads to activation of the MAPK and PI3K/AKT/mTOR signaling pathways that promote cell survival. PI3K downstream signaling involves conversion of PIP2 to PIP3, which recruits AKT and PDK1 to the plasma membrane. PDK1 and mTORC2 phosphorylate AKT, resulting in full activation of AKT. PTEN negatively regulates AKT through dephosphorylation of PIP3. AKT activity also results in anti-apoptotic signaling by phosphorylating pro-apoptotic protein BAD. Cisplatin-induced apoptosis is diminished by MDM2 binding to p53, which promotes p53 ubiquitination and degradation, resulting in lower p53 mediated transcriptional regulation of pro-apoptotic proteins.

provide evidence for altered levels of ERCC1 or other NER-related proteins to be involved in cisplatin resistance of TC. As a reduced ability to perform HR has been shown to determine cisplatin sensitivity in many cancers, changes in HR efficacy could determine cisplatin resistance in TC as well. In a study where cisplatin sensitive and resistant TC cell lines were compared, it was shown that cisplatin sensitivity correlates with the ability of cells to repair interstrand cross-links [48]. Despite differences in HR proficiency between cisplatin sensitive and resistant cells, all TC cell lines were shown to be more defective in HR when compared to a non-TC cisplatin resistant cell line [48]. Furthermore, an investigation into the presence of activated ATM has demonstrated that TC tumors show more activated ATM, as judged by serine 1981 phosphorylation, compared to normal tissue. However, the extend of ATM activation was less prominent compared to other tumor types [63]. These data further support the idea that TC tumors are characterized by a lower proficiency to activate the DDR machinery, but that there is no support of its involvement in cisplatin resistance.

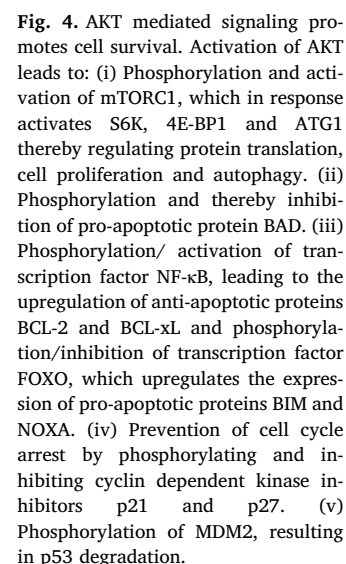
Embryonal carcinoma cell lines and tumors, like embryonic stem cells, are characterized by high expression of the pluripotency markers OCT4 and NANOG [64,65]. Several studies have demonstrated a link between loss of OCT4 expression and lower NOXA levels, which correlated with chemoresistance in vitro and in vivo [57,66–68]. More recently, a study showed chemotherapy induced depletion of OCT4 in a mouse model of TC including teratoma and EC components [69]. Similarly, loss of pluripotency markers OCT4 and NANOG was observed in a clinical cohort of TC with emerging chemotherapy resistance [30]. In addition, cisplatin treatment itself induced chemoresistance, as demonstrated in a study by Abada & Howell, where they showed that cisplatin treatment induced differentiation of EC cells in vitro, associated with a loss of pluripotency markers OCT4 and NANOG [70]. In

vitro differentiation of EC cells by retinoic acid also led to repression of OCT4, downregulation of PUMA and NOXA, and concomitantly a decreased sensitivity to cisplatin [56,71,72]. Combined, these data suggest that cisplatin-treated EC tumors which downregulate OCT4 expression become chemoresistant, either via subsequent lower NOXA levels or via differentiation of TC cells. Important to mention is that other TC subtypes, like YSC and CC, do not express OCT4, while these TC subtypes are very sensitive to cisplatin as well. Also clinically, no correlation has been demonstrated between OCT4 expression and cisplatin sensitivity [73].

As mentioned above, genomic alterations affecting the MDM2/p53 axis have been described in resistant TC. A large study examined 180 TC tumors using whole exome sequencing and described that most *MDM2/TP53* alterations were found in cisplatin resistant tumors [32]. The majority of *MDM2* amplifications were observed in post-treatment specimens suggesting that tumor cells are highly selected during treatment, a finding that is further supported by a recent study showing amplification of 12q15 containing *MDM2* in a refractory TC patient [32,34]. In TC cells it was shown that p53 function is hampered by the interaction with MDM2, especially in cisplatin-resistant cells, where a higher cisplatin concentration was needed to interfere with the MDM2/p53 interaction [53]. Therefore, MDM2 appears an interesting therapeutic target to activate WT p53. Indeed, small molecule inhibitors of MDM2 (e.g. nutlin-3a) targeting the MDM2-p53 interaction were shown to sensitize TC cells to cisplatin treatment [53,74]. Even though most TC tumors retain WT *TP53*, posttranscriptional modifications are able to repress the pro-apoptotic activity of p53. For example, lysine methylation at the carboxyl terminus of p53 repressed its transcriptional activity, as demonstrated by reduced expression of PUMA and p21 in TC cells [75]. Furthermore, the expression of miR-372 and miR-373 was

Recently, the PI3K/AKT/mTOR pathway has gained more attention in relation to cisplatin resistance of TC. Besides mutational deregulation, hyperactivation of the PI3K/AKT/mTOR pathway has been described. A central player in the PI3K/AKT/mTOR pathway is AKT, which functions as a pro-survival factor by promoting cell survival and inhibiting apoptosis (Fig. 4). Phosphorylated AKT has several ways to negatively regulate apoptosis. Firstly, AKT phosphorylates and thereby inhibits the pro-apoptotic protein BAD [81]. In addition, through direct inhibition of FOXO3a, which is responsible for the transcription of several pro-apoptotic proteins including PUMA and FAS ligand, AKT indirectly negatively influences apoptosis [82]. Secondly, AKT activates the transcription factor NF- κ B, which promotes the transcription of several anti-apoptotic genes including BCL-2 and BCL-XL [83,84]. Thirdly, AKT phosphorylates and activates mTORC1 which is a key regulator of protein translation, cell proliferation and autophagy [85]. Finally, it has been described that AKT-mediated phosphorylation of MDM2 promotes its nuclear localization, where it interacts with p53 and thereby targets p53 for degradation [86]. Altogether, these studies emphasize the involvement of AKT in diverse tumorigenic activities, summarized in Fig. 4. The first proof of PI3K/AKT/mTOR pathway hyperactivity in TC was provided by Di Vizio *et al.* who discovered that the majority of TC tumors are characterized by loss of PTEN, a tumor suppressor that negatively regulates PI3K signaling [87]. Other reports

Several important pro-apoptotic members and their role in apoptosis signaling have been discussed above, including the FAS death receptor and PUMA. Less known in the context of TC are the X-linked inhibitor of apoptosis (XIAP) and the IAP antagonistic protein SMAC/Diablo. XIAP is an important determinant of the apoptotic process and functions by inhibiting the initiator caspase-9 and effector caspases-3 and -7 [93]. SMAC/Diablo is released from the mitochondria during the apoptotic process, and promotes caspase activation by binding to and inhibiting XIAP [94,95]. Clearly, a shift in balance between XIAP and SMAC/Diablo may mediate apoptotic resistance, either by overexpression of XIAP, or by downregulation of SMAC/Diablo. One study examining the mRNA expression of XIAP and SMAC/Diablo in normal testis tissue and TC showed that XIAP levels were similar between normal and TC tissue, while the levels of SMAC/Diablo were decreased in TC compared to normal tissue [96]. These data further stress that the balance between pro- and anti-apoptotic proteins in TC is key in



controlling the apoptotic response. Interfering in this balance by blocking anti-apoptotic proteins might be a way to shift the balance in favor of apoptosis. For instance, BH3 mimetics block multiple or specific anti-apoptotic BCL-2 family members thereby inhibiting the activity of these pro-survival proteins and favoring apoptosis. Similarly, it would be interesting to test SMAC mimetics in TC models.

Novel preclinical models in TC

Despite the fact that TC is a complex and heterogeneous disease, TC models, including cell lines and xenografts, are scarce. Only ~20 human and mouse cell lines and less than 10 xenograft models have been reported [97–106]. Available cell lines and xenografts almost exclusively cover the EC subtype, highlighting the obvious need for more models representing other histological TC subtypes.

Patient-derived xenograft models are increasingly used in current cancer research due to several advantages over cell line-based xenografts, including maintenance of tumor heterogeneity, high similarity to human tumors [107,108] and increased predictive power of drug response [109,110]. So far, only 14 orthotopically established TC PDX models and 14 subcutaneous TC PDX models have been reported [92,111,112], with limited data available of their establishment [113]. One study described the establishment of 14 non-seminoma PDX models, specifically from the CC, EC and YSC subtypes, as well as mixed tumors with YSC, teratoma and EC components [113]. In this study, orthotopic implantation of the tumor pieces was more successful than subcutaneous implantation. These PDX models were used to study cisplatin resistance and to test novel combinatorial strategies using a glucosylceramide synthase (GCS) inhibitor, DL-threo-PDMP, and a multi-targeted receptor tyrosine kinase inhibitor, sunitinib, in combination with cisplatin [113,114]. Recently, we established and characterized three subcutaneous TC PDX models. Two models were obtained from a cisplatin-sensitive patient, and one model was obtained from a cisplatin-resistant patient. These PDX tumors and matched patient tumors showed retention of histological subtypes, were molecularly stable over several passages, and their chemosensitivity corresponded with patients' response to chemotherapy (de Vries *et al.*, in preparation). A broad panel of TC PDX models representing all histological subtypes, including tumors with a mixed histology, would be valuable to gain more understanding of the differential molecular make-up of histological subtypes. Differential expression of certain drug targets between histological subtypes, as demonstrated for CD30 and PDGFR β [89,115], might drive the development of treatments tailored to specific TC subtypes.

Novel targeted therapies in TC patients

First line cisplatin-based chemotherapy will fail in 10–15% of patients with metastatic disease, including refractory and relapsed patients [12]. These patients will receive salvage treatment, as described above, with varying success rates around 50% [5]. No alternative treatment options are available for TC patients not being cured by salvage regimens, highlighting the need for clinical trials investigating novel therapies. However, initiation and design of large clinical trials are difficult due to the low incidence and high cure rates of TC. Nonetheless, some clinical trials investigating targeted therapies have been performed and will be discussed below, as well as ongoing (pre-) clinical developments (Table 2).

One pre-clinical target that has shown promising results in several TC cell lines, is MDM2. Blocking the interaction between MDM2 and p53 by nutlin-3, a small molecule inhibitor of MDM2, induced apoptosis [53,74]. In addition, synergistic effects on cell survival were observed in TC cell lines after combined nutlin-3 and cisplatin treatment [53]. Several MDM2 inhibitors, including RG7388, AMG-232 and ALRN-6924, are currently under investigation in phase I-II trials. No clinical studies specifically investigating the potential of MDM2 inhibitors in

refractory or relapsed TC patients have been initiated to date.

Another pathway that is currently being investigated as a clinical target for treatment of TC is the DDR pathway. As TC tumors are characterized by defective repair of DSBs, targeting DDR components provides a therapeutic opportunity. This option has gained more attention, since HR-deficient tumors were shown to be highly sensitive to PARP1 inhibitors [116–118]. In vitro data has shown that treatment of TC cells with the PARP inhibitor olaparib sensitized these cells to cisplatin treatment, in particular cisplatin-resistant cells [48]. Expression of PARP1 has also been investigated in TC tumors, showing high expression in tumor tissue but not in normal testis tissue [119]. Currently, two phase II trials are evaluating the potential of PARP inhibitors in relapsed or refractory TC, either as single agent (NCT02533765, currently active) or combined with gemcitabine and carboplatin (NCT02860819, currently recruiting).

Another clinical target that has previously been investigated is CD30, a member of the TNF receptor family, which is expressed by the EC subtype of TC [115]. Interestingly, TC patients with CD30-expressing tumors had worse progression free survival and overall survival compared to patients with CD30-negative tumors [120]. Brentuximab-vedotin is an anti-CD30 antibody linked to the antimetabolic agent monomethyl auristatin E. This drug is FDA-approved for treatment of Hodgkin lymphoma, anaplastic large cell lymphoma and cutaneous T-cell lymphoma. A phase II clinical trial has investigated the therapeutic potential of brentuximab-vedotin in TC. This study included five patients with non-seminoma TC tumors of which one patient achieved a complete response and one patient a partial response [121]. However, another phase II trial in 18 relapsed and refractory non-seminoma TC patients, investigating the activity of brentuximab-vedotin, was recently terminated due to lack of efficacy (NCT02689219). This study had two arms, patients with CD30 negative/unknown tumors and patients with CD30 positive tumors. None of the patients achieved a partial or complete response, and the majority of patients showed disease progression. The lack of efficacy might be caused by acquired or intrinsic resistance to brentuximab-vedotin, such as loss of CD30 expression as described previously [121].

Recently it was shown that cisplatin resistant TC cell lines express higher mRNA and protein levels of the aldehyde dehydrogenase (ALDH) isoform ALDH1A3 compared to their cisplatin sensitive parental cells. Treatment with the ALDH inhibitor disulfiram sensitized TC cells to cisplatin treatment in vitro and in vivo. In addition, a significantly higher expression of the ALDH isoform ALDH1A3 was detected in TC tumors compared to healthy testicular tissue [122]. Based on these data, a phase II clinical trial will commence and evaluate the potential of disulfiram in combination with cisplatin in relapsed or refractory TC patients (NCT03950830, currently recruiting).

Claudin 6 (CLDN6) is a tight junction membrane protein, which was found to be aberrantly expressed in TC tissue while little expression was observed in normal tissue [123]. The tumor specific expression makes CLDN6 an attractive drug target. For that purpose the anti-CLDN6 monoclonal antibody ASP1650, also known as IMAB027, was developed. Pre-clinical data in TC cell lines showed that ASP1650 induced cell death as a single agent, and that pretreatment with chemotherapeutic agents upregulated CLDN6 expression in heterogeneously expressing cell lines [123]. A first-in-human trial was performed in advanced ovarian cancer patients, demonstrating that ASP1650 was safe and well tolerated [124]. A phase II clinical trial will now investigate the safety and efficacy of ASP1650 in refractory TC patients (NCT03760081, currently recruiting).

Research into the epigenetics of TC has suggested that increased DNA methylation is associated with cisplatin resistance. Cisplatin-sensitive seminomas were shown to be hypomethylated, while EC tumors that have higher incidence of cisplatin resistance showed intermediate DNA methylation. Treatment-resistant tumors including YSC, CC and teratomas showed higher levels of DNA methylation when compared to other solid tumors [125]. In line with this notion, TC cell lines were

Table 2
Finished and ongoing clinical trials in TC patients.

Target	Drug	Trial phase	NCT identifier	Status
PARP	Olaparib	Phase II	NCT02533765	Active
PARP + DNMT	Veliparib + Gemcitabine + Carboplatin	Phase II	NCT02860819	Active and recruiting
CD30	Brentuximab-vedotin	Phase II		Completed [121]
CD30	Brentuximab-vedotin	Phase II	NCT02689219	Terminated, lack of efficacy
ALDH	Disulfiram + cisplatin	Phase II	NCT03950830	Recruiting
CLDN6	ASP1650	Phase II	NCT03760081	Recruiting
DNMT	Guadecitabine (SGI-110) + cisplatin	Phase I	NCT02429466	Active
PDGFR + VEGFR + KIT	Sunitinib	Phase II		Completed [127]
		Phase II		Completed [128]
PDGFR + VEGFR + KIT	Pazopanib	Phase II		Completed [129]
KIT + PDGFR + BCR-ABL	Imatinib	Phase II		Terminated, lack of efficacy [130]
		Phase II		Terminated, lack of efficacy [131]
mTORC1	Everolimus	Phase II		Completed [132]
		Phase II		Completed [133]
mTORC1 + EGFR	Sirolimus + Erlotinib	Phase II	NCT01962896	Terminated, low accrual
CDK4/6	Palbociclib	Phase II		Completed [134]
CDK4/6	Ribociclib	Phase II		Completed [136]
PD-1	Pembrolizumab	Phase II		Terminated, lack of efficacy [142]
PD-1 + CTLA-4	Nivolumab + Ipilimumab	Phase II	NCT03333616	Recruiting
PD-L1	Avelumab	Phase II		Completed [143]
PD-L1 + CTLA-4	Durvalumab + Tremelimumab	Phase II	NCT03158064	Recruiting
PD-L1 + CTLA-4	Durvalumab +/- Tremelimumab	Phase II	NCT03081923	Recruiting

PARP: poly(ADP) ribose polymerase; DNMT: DNA methyl transferase; ALDH: aldehyde dehydrogenase; CLDN6: claudin 6; PDGFR: platelet-derived growth factor receptor; VEGFR: vascular endothelial growth factor receptor; KIT: stem cell growth factor receptor; mTORC1: mammalian target of rapamycin complex 1; EGFR: epidermal growth factor receptor; CDK4/6: cyclin-dependent kinase 4/6; PD-1: programmed death receptor 1; PD-L1: programmed death ligand 1; CTLA-4: Cytotoxic T-Lymphocyte Associated Protein 4.

proven to be hypersensitive to the second generation DNA methylation inhibitor guadecitabine (SGI-110), both in vitro and in vivo. In addition, pretreatment of cisplatin resistant TC cells with this inhibitor re-sensitized cells to cisplatin [98]. A phase I clinical trial will evaluate the safety and efficacy of guadecitabine in combination with cisplatin in refractory TC patients (NCT02429466, currently active).

Several receptor tyrosine kinases (RTK) including KIT, ERBB2, PDGFR and VEGFR are involved in activation of the MAPK and PI3K/AKT/mTOR pathway in TC [126]. Sunitinib, pazopanib and imatinib are multi-targeted inhibitors of RTKs including VEGFR, PDGFR and KIT that showed promising activity in vitro and in vivo using orthotopic PDX models of the YSC and CC histological subtypes [90,114,127]. Results of clinical trials, however, have been disappointing, with sunitinib only showing modest activity in one study, which was not confirmed in a second trial [127,128]. The trial investigating pazopanib showed no objective responses but achieved progression free survival of at least 3 months in 13% of patients [129]. Two other small phase II clinical trials tested the efficacy of imatinib in heavily treated TC patients. Six patients with mutated *KIT* were treated with imatinib showing poor results with 5 out of 6 patients developed progressive disease [130]. In the other clinical trial, none of the patients with KIT overexpression responded [131].

In vitro and in vivo efficacy of downstream targeting of the PI3K/AKT/mTOR pathway has been demonstrated in TC models, showing increased apoptosis and tumor growth inhibition [68,92]. Two phase II clinical trials have tested the mTORC1 inhibitor everolimus in refractory or relapsed TC patients. Efficacy of everolimus was limited in both trials reporting no objective responses, but one study reported 12-week progression free survival in 40% of patients [132,133]. One other small phase II clinical trial tested the mTORC1 inhibitor sirolimus in combination with EGFR inhibitor erlotinib but closed prematurely due to lack of efficacy (NCT01962896). Summarizing, major clinical outcomes in response to sunitinib, pazopanib, imatinib and everolimus were limited to case reports and small phase II studies and can therefore not be recommended as single agent treatment [127–133]. These clinical studies used single drug treatment to test efficacy. However, several pre-clinical studies have shown that combining targeted agents with cisplatin enhances therapeutic efficacy in TC models. For example,

in a recent study we showed that mTORC1/2 inhibitors sensitized TC cell lines to cisplatin treatment more strongly than everolimus, an mTORC1 inhibitor. Everolimus treatment prompted an upregulation of AKT phosphorylation levels, resulting from a positive feedback loop between mTORC2 and AKT. This feedback loop might explain the reduced efficacy of everolimus in clinical setting. Importantly, synergistic effects were observed between mTORC1/2 inhibitor AZD8055 and cisplatin in TC cell lines. Furthermore, AZD8055 potentiated the efficacy of cisplatin in two TC PDX models, one pure YSC and one mixed tumor consisting of YSC and immature teratoma components [92]. Concluding, combined targeted treatment with cisplatin hold potential for future clinical development in TC patients.

Patients with teratoma usually undergo surgery to remove residual metastatic lesions. However, there are no alternative treatments for patients with teratomas that are unresectable or progressive. Teratomas express high levels of the tumor suppressor retinoblastoma protein (Rb), unlike less differentiated TC tumors i.e. seminomas and EC [134]. Rb protein is an important regulator of the G1-S checkpoint, blocking entry into S-phase and promoting cell differentiation [135]. Cyclin-dependent kinases 4 and 6 (CDK4/6) in association with cyclin D phosphorylates Rb, leading to cell cycle progression. The inhibition of CDK4/6 is therefore an attractive therapeutic target in tumors expressing high levels of Rb. Palbociclib is a CDK4/6 inhibitor that has been evaluated in a phase II study in refractory TC patients with Rb-expressing tumors. Of the 29 patients included, eight achieved progression free survival of 24 weeks. In addition, patients with teratoma or teratoma with malignant transformation had significantly better progression free survival compared to patients with other histological TC tumor types [134]. Recently, a randomized placebo controlled phase II trial investigating the CDK4/6 inhibitor ribociclib was reported in which TC patients with unresectable and progressive teratoma without malignant transformation were included. Due to slow accrual this trials was prematurely closed. Eight patients were included in the ribociclib group which showed a progression free survival rate of 71% at 24 months, indicating some anti-tumor activity of this compound for this indication [136].

While immunotherapy has proven beneficial in many tumor types [137], limited research has been performed in TC which might be due

to the fact that testes are regarded as immune privileged. Two studies have looked into the expression of PD-L1 in TC tissue, and both reported a higher expression of PD-L1 in tumor compared to normal testis tissue [138,139]. Percentages of tumors positive for PD-L1 ranged between 73–76% for seminomas and 64–89% for non-seminomas. PD-L1 expression on tumor cells can serve as a predictive marker for response to anti-PD-1 or anti-PD-L1 immunotherapy in several tumor types [137,140,141]. Indeed, low PD-L1 expression in TC tumors was associated with a significantly better progression-free survival [139]. Two small phase II studies have evaluated immune checkpoint inhibition in refractory and relapsed TC patients. No objective responses were observed with single agent pembrolizumab or avelumab indicating lack of efficacy in unselected patient groups [142,143]. A noteworthy observation from the pembrolizumab study was the fact that only two of the 12 patients had PD-L1 positive tumors. Another possible explanation for the lack of clinical efficacy is the low mutational burden in TC tumors [27] and possibly low number of neoantigens. In addition, whereas extensive immune infiltration has been shown for seminomas, this was considerably lower for non-seminomas [29]. Regardless, several clinical studies will commence and investigate the efficacy of immunotherapy in TC. Two phase II trials will investigate the combination of anti-PD-L1 inhibitor durvalumab with anti-CTLA-4 inhibitor tremelimumab in refractory TC patients (NCT03081923, NCT03158064, both currently recruiting). The safety and efficacy of another immunotherapy combination, anti-PD-1 inhibitor nivolumab combined with anti-CTLA-4 inhibitor ipilimumab, will be investigated in a phase II trial in refractory TC patients (NCT03333616, currently recruiting).

Overall, results of clinical trials in TC patients investigating targeted drugs have been disappointing. Some limitations in these studies that might have influenced the clinical outcomes are: (1) patients were heavily pretreated, which has to do with the fact that there are several salvage treatment options available with excellent outcomes, (2) inclusion of small patient numbers and (3) the large heterogeneity of TC subtypes included. Unfortunately, these limitations are not easily solved for future clinical trials in TC patients. Nevertheless, clinical trials investigating novel targeted agents for TC are underway, including trials investigating a targeted agent combined with chemotherapy (NCT02860819, NCT03950830, NCT02429466).

Concluding remarks

Excellent sensitivity of TC tumors to cisplatin treatment has made TC a highly curable disease. Two features of TC tumors contribute to cisplatin sensitivity, and are described here: insufficient DNA repair in response to cisplatin-induced DNA damage, and a hypersensitive apoptotic response. However, even though TC is a highly curable disease there are some therapeutic challenges left.

One important issue affecting survival of TC patients is cisplatin resistance. Unfortunately, there are no alternative treatment options for patients who relapse and do not sufficiently respond to salvage treatment or those that are refractory. Some mechanisms contributing to cisplatin resistance have been identified including hyperactivity of the PI3K/AKT/mTOR pathway and elevated levels of MDM2. Following, several strategies to overcome cisplatin resistance have shown efficacy in vitro and in vivo. These strategies include combinations of cisplatin with DNA methylation inhibitors, PARP inhibitors, MDM2 inhibitors, and PI3K/AKT/mTOR inhibitors. However, despite the current knowledge on the molecular mechanisms underlying TC biology and the identification of successful pre-clinical targeted approaches, no targeted drugs have hitherto shown robust clinical benefit. Following pre-clinical results, it seems logical that future clinical trials should focus on combining targeted drugs with cisplatin in an effort to overcome cisplatin resistance. In addition, histological subtypes should be taken into account, as demonstrated for the CDK4/6 inhibitor trials in teratoma. Excitingly, three clinical trials are currently recruiting or active that will investigate the combination of chemotherapy with a targeted drug,

i.e. veliparib, guadecitabine and disulfiram. Furthermore, given the hypersensitive apoptotic response of TC cells associated with high mitochondrial priming, one could speculate that the break on apoptosis present in cisplatin-resistant cells can be removed by the addition of BH3 mimetics or SMAC mimetics. Several BH3 mimetics and SMAC mimetics are in clinical development and warrant further investigation using in vivo TC models.

Author contributions

All authors conceived the study. G.V. and X.R. performed the literature search and wrote the manuscript. M.A.T.M.V., J.A.G. and S.J. supervised, reviewed and edited the manuscript. All authors discussed the results and commented on the manuscript.

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Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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